DNA Complexes

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DNA-Controlled Bivalent Presentation of Ligands for the Estrogen Receptor**

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The assembly of DNA complexes proceeds according to known rules. Thus, the mutual recognition of DNA conjugates can be used for the precise positioning of functional groups. For example, chromophores,[1] metals,[2] catalytic units,[3] nanoparticles,[4] fluorophores[5] and even proteins[6] have been arranged at well-defined distances by means of DNA hybridization. Until recently, the main interest was focused on issues within materials science as well as on the immobilization of biomolecules. We and others assumed that the ability to position functional units at defined distances could also be used to address biological problems.[7] According to this, DNA may serve as a molecular ruler to determine the distance between binding pockets in biological receptors. Due to self-assembly of the DNA complex the rapid spatial screening of a receptor can be doen with minor synthetic effort.[8] In this approach, the ligand of a biological receptor is covalently attached to an oligonucleotide (Figure 1 a). The binding of two or more oligonucleotide–ligand conjugates to a template strand provides bi- or multivalent DNA–ligand conjugates. The distance between the two biologically active ligands can be readily adjusted by varying of the template strand. Herein we demonstrate, for the first time, the DNA-controlled presentation of small molecules in the spatial screening of a protein receptor. We demonstrate the advantages conferred by DNA spacers by examining a well-studied nuclear receptor, the estrogen receptor, and by comparison with commonly applied oligoethyleneglycol spacers.

The estrogen receptor (ER) is activated by the hormone estradiol and is involved in the regulation of gene expression.[9] It is assumed that the formation of dimers is essential for the natural function of the receptor (Figure 1 b).[10] The dimerization constant is in the subnanomolar range, yet it must be considered that ligand binding can influence the dimerization equilibrium.[11] The selective estrogen receptor modulators (SERMs) hexestrol, raloxifene,[12] and 4-hydroxytamoxifene[13] stabilize the receptor dimer and were thus deemed suitable for the spatial screening of the ER.[10a]

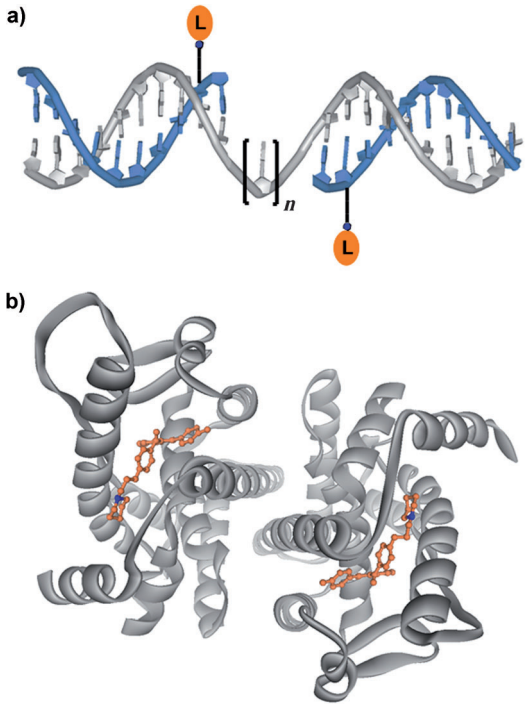

Figure 1. a) Bivalent presentation of estrogen receptor ligands (L) on ternary DNA complexes. b) Crystal structure of the ligand binding domain of the estrogen receptor (PDB ID: 1ERR) in complex with raloxifene (orange). The nitrogen atoms of raloxifene (blue) are 35 Å apart.

The synthesis of the SERM–oligonucleotide conjugates was achieved by introducing the alkyne-modified uridine building block **X** during automated DNA synthesis (Scheme 1). The SERMs were equipped with azido functions[14] to enable the covalent attachment to the oligonucleotides **ODN-X** by the Cu-catalyzed 1,3-dipolar cycloaddition.[15] The resulting conjugates **ODN-XR** were obtained in 30–70 % yield.

The affinity of the oligonucleotide–SERM complexes to the estrogen receptor (ER-α) was assessed by means of the HitHunter assay.[16] The conjugation of hexestrol (Hex) with an oligonucleotide diminished the affinity by several orders of magnitude (Figure 2). This result appears plausible because in the structure of the ER in complex with agonists such as hexestrol the binding pocket is nearly closed (Figure S33 in the Supporting Information). In contrast, the estrogen analogues raloxifene (Ral) and 4-hydroxytamoxifene (Tam)

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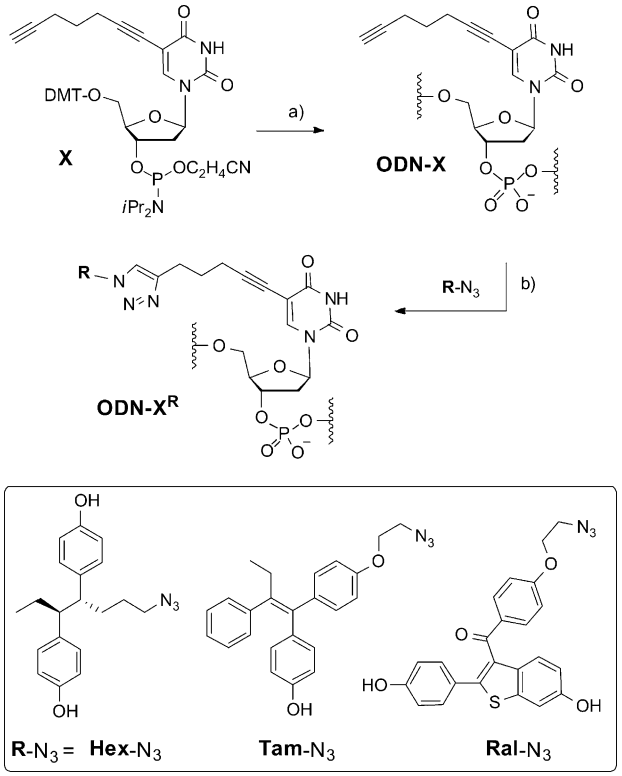

Scheme 1. Synthesis of oligonucleotide–SERM conjugates. a) DNA synthesis (DMT = 4,4′-dimethoxytrityl); b) 1,3-dipolar cycloaddition: Cu_2SO_4, 1–10 equiv azide, tris(hydroxypropyl)triazolyl methyl amine (THPTA), sodium ascorbate, NaCl, urea, H_2O/DMSO, 2 h, 80 °C.

showed high ER-binding affinity even after conjugation to DNA in **2Tam** and **2Ral**. Since the ER is a transcription factor, it is conceivable that contributions of the oligonucleotide scaffold lead to increases in the affinity of the conjugates for the ER. This would explain why conjugate **2Ral** binds the ER with higher affinity than free raloxifene. Variation of the nucleic acid sequence (in **3Ral**) led to markedly reduced binding affinities, and it is unlikely that ionic interactions with the DNA backbone are the sole reason for the high ER affinity of **2Tam** and **2Ral**. Control experiments suggested that unmodified oligonucleotides failed, within the limits of the binding assay, to bind to the ER (Figure S32 and Table S4 in the Supporting Information).

The spatial screening of the ER binding pockets was performed using self-assembled complexes in which two different 4-hydroxytamoxifene–ODN conjugates or two different raloxifene–ODN conjugates were annealed to a template strand. The distance between the ligands was varied by changing the number of the unpaired template nucleotides Y_n in the formed bivalent, ternary complexes $\mathbf{4R}_n$ (Figure 2). Melting studies proved that the conjugation of ER ligands affected the stability of ternary complexes **4** to a negligible extent (Figures S18–S20 in the Supporting Information). The binding experiments revealed a remarkably high level of relative binding affinities (RBA) of up to 300% RBA. This result is noteworthy because earlier studies which involved flexibly linked SERM dimers had shown relatively low binding affinities, RBA ≤ 7%.[17]

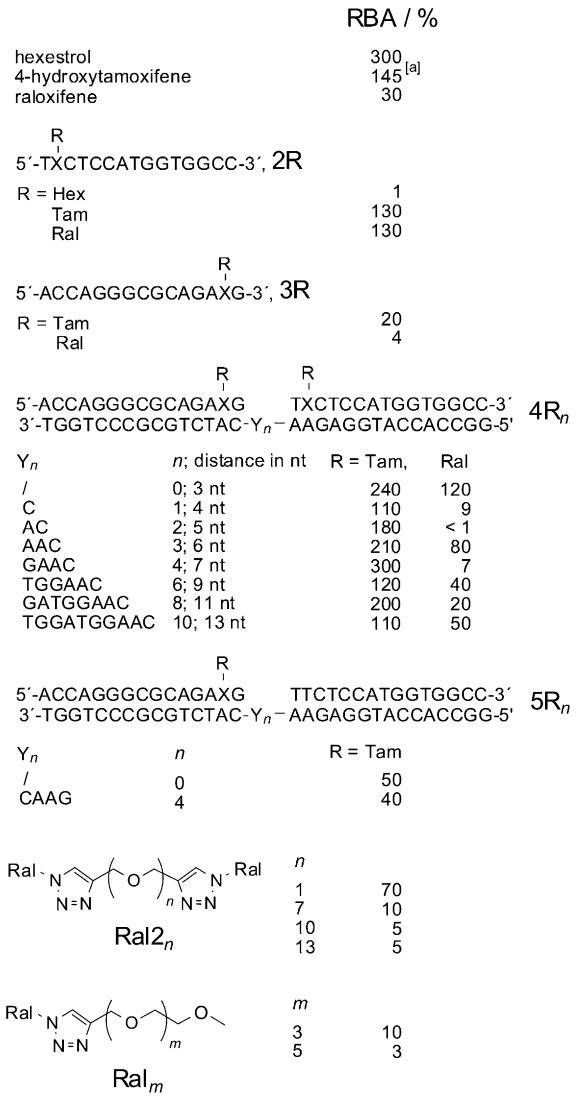

Figure 2. Relative binding affinity (RBA, relative to estradiol). [a] Calculated for a 1:1 mixture of the *cis*/*trans* isomers. nt = nucleotide. (Conditions: see Table S4 in the Supporting Information.)

As a control, bivalent complexes $\mathbf{4R}_n$ were compared with monovalent complexes $\mathbf{5R}_n$, which comprise the same DNA architecture. This comparison revealed the advantages of bivalent presentation. The bivalent complexes $\mathbf{4Tam_0}$ and $\mathbf{4Tam_4}$ showed five to seven times higher affinity to the ER than the monovalent complexes $\mathbf{5Tam_0}$ and $\mathbf{5Tam_4}$. The highest affinity was determined for complexes in which the 4-hydroxytamoxifene units were separated by three ($\mathbf{4Tam_0}$) or seven ($\mathbf{4Tam_4}$) nucleotides. A similar result was obtained in the evaluation of the raloxifene conjugates, where the affinities of bivalent constructs exceeded the affinity of the monovalent conjugates. The distance dependence was even more pronounced. Again, two maxima of the binding affinity were observed at a separation of three ($\mathbf{4Ral_0}$) and six ($\mathbf{4Ral_3}$) nucleotides.

It is significant that both 4-hydroxytamoxifene–DNA and raloxifene–DNA conjugates showed the highest binding

affinities when arranged three, six, or seven nucleotides apart, respectively. For an estimation of the distances it was assumed that the complexes adopted the structure of B-DNA (20 Å helix diameter, 3.4 Å base-to-base distance, 10.2 nucleotides per turn). This approximation is justified because it has been shown that fully base-paired ternary complexes such as **4Ral$_0$** and **4Tam$_0$** maintain the structural characteristics of B-DNA.[18] Furthermore, we ascertained in accompanying FRET studies (FRET = fluorescence resonance energy transfer) that the length of a ternary complex that contains two double-helical segments separated by three unpaired template nucleotides concurs with the length of a canonical B-duplex.[8b]

Based on this estimation and taking into account the linker length and the helical torsion, it was concluded that a three-nucleotide spacer will arrange the triazole units at a distance of less than 23 Å. A spacer six or seven nucleotides long will position the triazole units 38–40 Å apart. It should be considered, however, that the unpaired nucleotides will increase the flexibility of ligand presentation. This distance is within sufficient agreement with the 35 Å calculated for the distance between the nitrogen atoms of raloxifene in a co-crystal with the ER-α dimer.[19]

Figure 3a shows the result of a docking study which suggests that the DNA complexes can indeed bridge the distance between the protein binding pockets. In addition, molecular dynamics calculations point to the semirigid character of ternary DNA complexes. According to the calculations, the length dimension of a DNA helix is maintained even when unpaired nucleotides are included (Figure S45 in the Supporting Information). While double-stranded segments adopt a rodlike structure, segments that contain unpaired nucleotides convey local flexibility to allow for torsion and bending (Figure S46 in the Supporting Information). As a result, the variance of distances and torsion angles is higher when the two modified nucleotides are part of a ternary complex that involves unpaired nucleotides than when they are part of a contiguously base-paired DNA duplex. It is difficult to imagine that the **4Ral$_0$** complex can fit the ligands into the consensus binding pockets of the ER dimer.

A recent study showed that the ER-β dimer can bind not only two but also four 4-hydroxytamoxifene molecules.[20] The second binding pocket is in the immediate vicinity of the consensus binding pocket, which places the nitrogen atom of the additionally bound 4-hydroxytamoxifene derivatives at a distance of 17 Å away from the nitrogen atom of the first ligands. For ER-α it has been shown that hydrophobic peptides can bind to an adjacent, hydrophobic coactivator binding pocket.[21] It is conceivable that this binding site is used by one of the two SERMs in complexes such as **4Tam$_0$** and **4Ral$_0$**. This assumption was examined in a docking study. One ligand of the DNA conjugates was docked into the consensus binding pocket of ER-α. Different binding states were modeled for the second ligand. The stability of these binding states was assessed in MD simulations. The interaction energy released upon interaction of the conjugate with the protein was characterized for different potential binding sites (Figure 3b, Figures S34–S40 in the Supporting Information).[22] This suggested that a second raloxifene group can bind to a hydrophobic area defined by helix 3 and helix 4 (Figure 3c).

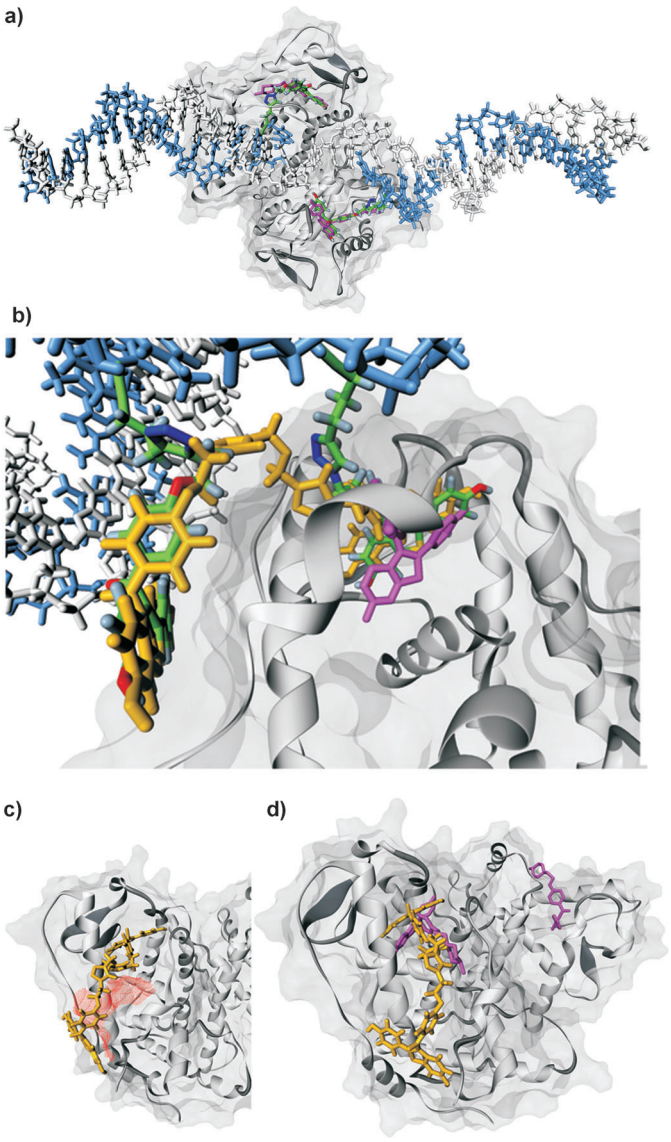

Figure 3. Docking of the ternary DNA complex a) **4Ral$_3$** and b) **4Ral$_0$** (green, conjugated raloxifene with spacer) as well as the ether-bridged raloxifene conjugate **Ral2$_1$** (yellow) to the ligand binding domain of ER-α (gray) and depiction of unconjugated raloxifene (magenta) in the co-crystal (PDB ID: 2R6W); c) depiction of the hydrophobic contact region (red) and **Ral2$_1$** (yellow); d) docking of the conjugate **Ral2$_1$** (yellow) with ER-α.

The results of the binding experiments and the docking study indicate the possibility that small hydrophobic molecules such as raloxifene and 4-hydroxytamoxifene can bind not only to the consensus binding pocket but also to a second hydrophobic site of the ER-α. Further support for this assumption was sought in binding affinities of ligand dimers **Ral2$_n$**, wherein the raloxifene units were linked by flexible oligoethyleneglycol structures in analogy to earlier studies (Figure 2).[17] Interestingly, the highest affinity for the ER-α was obtained with conjugate **Ral2$_1$** which contained the

shortest linker. The RBA of this bivalent conjugate is seven times higher than that of the monovalent raloxifen–oligoethyleneglycol conjugate **Ral$_3$**. Again, a docking study illustrated the potential of the simultaneous binding of the raloxifene units to the consensus binding pocket and an adjacent hydrophobic site (Figure 3b,d).

Additional experiments with compounds in which the raloxifene units were conjugated to hydrophobic groups such as pyrene suggested that the hydrophobic site can be occupied by "non-SERMs" (Table S4 in the Supporting Information, Ral-Pyr). In contrast, conjugates **Ral2$_7$**, **Ral2$_{10}$**, and **Ral2$_{13}$**, in which the raloxifene units were connected by flexible and long spacers, were found to bind with an affinity that only slightly surpassed the affinity of the monovalent conjugates **Ral$_3$** and **Ral$_5$**. This result is in agreement with earlier studies on flexibly linked SERM conjugates.[17b,d] As a control, we conducted experiments with raloxifene dimers in which single-stranded bivalent oligonucleotides served as flexible linkers. A previous study showed that the binding of bivalently modified DNA single strands to bivalent receptors should—in principle—lead to higher binding affinities than those expected for the binding of monovalent oligonucleotide conjugates.[8b,23] However, we observed that the bivalent single strands had a lower ER affinity than the monovalent single strands (Table S4 in the Supporting Information), which is in agreement with the results obtained with flexibly bridged ligand dimers.[17b,d]

At this stage of research, we can only speculate about the reason for the low binding affinity. It is conceivable that flexible linkers permit intramolecular, hydrophobic interactions between the SERM units, as was recently reported in a modeling study.[24] The binding of the ER to such conjugates would cost an energy penalty for the loss of the hydrophobic interactions between the SERMs. Previous work on contact quenching in fluorescence-labeled oligonucleotides has shown that two conjugated groups in a single strand can come into collisional contact.[25] In stark contrast, such an interaction is hampered when the conjugated groups are embedded within the rigid environment provided by double-helical segments. The α-estrogen receptor has frequently been probed with steroidal and nonsteroidal ligand dimers.[17] Oligomethylene-, oligoethyleneglycol-, and oligopropyleneglycol-bridged binders showed relative binding affinities of less than 7%. So far, it has been difficult to construct bivalent ligands that bind with higher affinity than monovalent ligands. Our data furnish evidence that high-affinity bivalent binders can be devised. In contrast to previous studies the ER ligands in the ternary DNA complexes **4R$_n$** are presented in a semirigid scaffold. In these complexes the ER ligands are bound to two rigid DNA-duplex segments and connected by means of a short, flexible hinge region. The DNA-programmed spatial screening showed that ER-α favors two assemblies. The fact that one of these arrangements (**4Ral$_3$**) positions the ligands at a distance that a) is also found in the crystal structure of the ER–raloxifene complex[19] and b) was identified by Katzenellenbogen et al. in studies of flexible linked estradiol dimers[17b] approves the method.

Moreover, the experiments indicate an additional optimum at short distances. We assume that this may point to a second hydrophobic binding site near the consensus ligand binding pocket of ER-α. This assumption is supported by docking studies and binding experiments, in which raloxifene was linked through short spacers to a second raloxifene or a hydrophobic group such as pyrene. The DNA-controlled ligand presentation should facilitate the spatial screening of receptors because this method involves the synthesis of only two ODN–ligand conjugates rather than the synthesis of a multitude of differently covalently conjugated dimers. This may accelerate the identification of high-affinity binders of structurally not characterized target proteins for biological and medicinal applications.

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